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# Recombinant DNA Molecules of Bacteriophage φX174

**(electron microscopy/spheroplast assay/multiple length DNA molecules/recombination models)**

ROBERT M. BENBOW*, ANTHONY J. ZUCCARELLI, AND ROBERT L. SINSHEIMER

Division of Biology, California Institute of Technology, Pasadena, Calif. 91125

*Contributed by Robert L. Sinsheimer, March 15, 1974*

**ABSTRACT** **φX174 DNA structures containing two different parental genomes were detected genetically and examined by electron microscopy. These structures consisted of two monomeric double-stranded DNA molecules linked in a figure 8 configuration. Such DNA structures were observed to be formed preferentially in host *recA*$^+$ cells or *recA*$^+$ cell-free systems. Since the host *recA*$^+$ allele is required for most φX174 recombinant formation, we conclude that the observed figure 8 molecules are intermediates in, or end products of, a φX174 recombination event.**

**We propose that recombinant figure 8 DNA molecules arise as a result of "single-strand aggression," are stabilized by double-strand "branch migration," and represent a specific example of a common intermediate in genetic recombination.**

Genetic recombination in bacteriophage φX174 (1, 2) and in the closely related bacteriophage S13 (3) has been analyzed extensively by both genetic (4–9) and physical (10–16) methods. Most φX174 recombinants are formed by a major pathway, Tessman's primary mechanism (4, 5), which requires the host *recA*$^+$ allele (6) but apparently does not need any of the nine known φX174 gene products (7). Recombinant formation via this major pathway involves two parental replicative form (RF) DNA molecules (5, 7), and occurs very early in the infection process (10). Single recombination events via the major pathway usually generate only one parental genotype and one recombinant (7). The goal of this work was to identify φX174 DNA structures which were formed by this major pathway.

To determine whether a particular DNA structure was recombinant, parental replicative form DNA molecules were isolated from cells infected with two parental genotypes (11). After purification and fractionation of the DNA molecules by velocity and equilibrium sedimentation procedures (12, 13), the frequency of recombinants associated with various DNA structures was examined genetically using a spheroplast assay in which further recombination could not occur (10). In addition, we were able to identify putative recombinant DNA molecules formed in mixed infections by using two parental genotypes which could be distinguished by electron microscopy (13).

In this paper we present electron micrographs of φX174 DNA molecules which apparently are recombinant. These structures appear to be "figure 8" molecules (15, 17) consisting of linked monomers of two double-stranded parental genomes. The existence of figure 8 molecules containing two parental genotypes supports the proposals of Doniger *et al.* (15, 18), and Benbow (10) that figure 8 DNA molecules are intermediates in the major pathway of φX174 recombinant formation.

We propose a simple mechanism to generate figure 8 DNA molecules that are recombinant. It is based on two molecular processes—"single-strand aggression" (11) and "branch migration" (19–21). These were previously implicated in recombinant formation in φX174 (10, 11, 13, 15) and in other organisms (20, 22).

## MATERIALS AND METHODS

Replicative form and multiple length DNA molecules were isolated from cells infected with two genotypes (9, 11, 13, 23; R. M. Benbow, M. Eisenberg, and R. L. Sinsheimer, to be published). The fractionated DNA molecules were examined for recombinants by carrying out spheroplast infections (24), after which the progeny phage were assayed for wild-type recombinants (9). To minimize recombination during the assay, spheroplasts were prepared from NH4547, an *Escherichia coli* K12 strain that is *recA*$^-$, *recB*$^-$, *uvrA*$^-$ (7, 10). It is important that the spheroplast assay strain contain both *recA*$^-$ and *recB*$^-$ alleles, and be φX174 resistant.

*Electron Microscope Assay for Recombinant DNA Molecules.* Mixed infections with *am3*(E) and *delE*$_{25}$ (13, 25), two genotypes shown previously (13) to be distinguishable by electron microscopy, were carried out. RF DNA molecules were prepared (11) and viewed by electron microscopy (26).

*Recombinant Formation in a Cell-Free System.* To assay for recombinant formation in a cell-free system, it was first necessary to prepare replicative-form DNA molecules that lacked recombinant DNA structures. One-liter cultures of HF4712 *recA*$^-$ were infected mixedly with *am3*(E) and *delE*$_{25}$ (multiplicity of infection $\cong$ 5 for each genotype), and replicative-form DNA molecules were prepared as described by Benbow *et al.* (11). Regions containing RF II DNA molecules (16 S) and some RF I DNA molecules (up to one fraction *before* the peak of RF I) were pooled from preformed CsCl gradients, concentrated, and dialyzed.

To carry out the assays, cell-free sonicates of *recA*$^+$ cells and of *recA*$^-$ cells were prepared. One-liter cultures of HF714 (*recA*$^+$) or HF4712 (*recA*$^-$) were grown in KC broth (9) at 37° with aeration to a concentration of 5 × 10$^8$ cells per ml. Cells were concentrated 20-fold by pelleting and resuspending in fresh KC broth containing 400 μg/ml of lysozyme, 0.05 M EDTA. After 20 min at 37° these were sonicated six times for 30 sec with a Branson sonicator (large tip). Cell breakage was

Abbreviation: RF, replicative form.

* Current address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge, England CB2-2QH.

TABLE 1. *Genetic assay of recombinant DNA molecules*

| Host | Structure assayed | Spheroplast assay | Recombination frequency (wild type/total phage) × $10^4$ |
|---|---|---|---|
| | Two-factor cross *am3*(E) × *am86*(A) | | |
| HF4714 | Direct burst of bacteriophage | None | 8.3 ± 0.9 |
| HF4704 | Direct burst of bacteriophage[1] | None | 9.7 ± 1.2 |
| HF4704 | RF I | NH4547 | 15.5 ± 1.7 |
| HF4704 | "26S" [2] | NH4547 | 29.6 ± 2.3 |
| HF4704 | "Catenanes" [3] | NH4547 | 40.3 ± 2.1 |
| HF4704 | "Interband Region" [4] | NH4547 | 90.7 ± 10.3 |
| HF4704 | "Circular" [5] | NH4547 | 27.2 ± 2.1 |
| | Two-factor cross *am3*(E) × *am9*(G) | | |
| HF4714 | Direct burst of bacteriophage | None | 6.8 ± 0.8 |
| HF4712 *recA* | Direct burst | None | 2.3 ± 0.4 |
| HF4714 | RF I | NH4547 | 11.4 ± 1.3 |
| HF4714 | "26S" [2] | NH4547 | 19.8 ± 2.1 |
| HF4712 *recA* | RF I | NH4547 | 3.5 ± 0.8 |
| HF4712 *recA* | "26S" [2] | NH4547 | 5.8 ± 2.7 |
| HF4714 | RF I, no chloramphenicol | NH4547 | 7.1 ± 0.6 |
| HF4714 | "26S",[2] no chloramphenicol | NH4547 | 13.3 ± 1.5 |

[1] Although HF4704 is nonpermissive, *am3*(E) is complemented by *am86*(A), which allows lysis; *am3*(E) grows efficiently even in nonpermissive cells, and the yield of progeny phage is normal.

[2] "26S" is the total population of multiple-length DNA molecules; it includes circular, catenated, and figure 8 DNA molecules.

[3] The middle band of a propidium bromide–CsCl gradient (Benbow, Eisenberg, and Sinsheimer, to be published); contains less than 10% circular dimers, 70% catenanes, and 20% figure 8's.

[4] The region between the middle and upper bands of a propidium bromide–CsCl gradient; contains 44% circular, 24% catenated, and 32% figure 8 molecules.

[5] The upper band of a propidium bromide–CsCl gradient; contains 61% circular, 27% catenated, and 12% figure 8 molecules.

estimated to be over 99% complete, and survivors were not detected in a $10^{-6}$ dilution.

To the 50 ml of sonicate was added the recombinant-free replicative-form DNA at a multiplicity of infection (based on $A_{260}$) of 10 RF of each genotype per initial cell. The cell-free mixture was incubated 30 min at 37°, shaken, centrifuged 6000 × *g* for 20 min to remove debris, and extracted directly with two volumes phenol (saturated with 0.05 M sodium tetraborate). RF DNA molecules were prepared as described (4) and were examined by electron microscopy. DNA structures were classified according to the criteria of Benbow, Eisenberg, and Sinsheimer (to be published).

## RESULTS

Rush and Warner have reported that *circular* multiple length DNA molecules of bacteriophage S13 were enriched 10- to 15-fold for recombinants relative to monomeric RF DNA molecules isolated from the same infection (12, 14). We were unable to confirm this result in φX174. Instead, as shown in Table 1, we have observed at most a 2-fold increase in recombinants in the total population of multiple length molecules. Furthermore, these recombinants did not segregate with circular dimers during equilibrium sedimentation in propidium bromide–CsCl (Table 1). Instead, a higher recombination frequency was observed in the band containing predominantly "*catenanes*"; a similar result has been obtained by Doniger *et al.* for S13 (15). Finally, we also have observed a high frequency of recombinants in regions of propidium bromide–CsCl gradients that do not correspond to any major band (Table 1).

These data support our previous conclusion (13) that most circular dimers are *not* recombinant, and that some molecules classified as "catenanes" contain two genotypes. These data also establish, as we emphasized previously, that not all catenanes are recombinants. (If all catenanes were recombinants, the catenane band would contain many fold more recombinants than the circular band; it does not, so they were not.)

We now propose that most, if not all, of the observed φX174 recombinants arise from fused dimers (27)—so-called figure 8 molecules (19). In our earlier work (13) using the electron microscope we classified figure 8 molecules with catenanes. However, true catenanes are topologically interlocked rings which usually show two crossover points under our spreading conditions. Figure 8 molecules show only one crossover point. Thus, our earlier report implicating catenanes in genetic recombination did not distinguish true from apparent (figure 8) catenanes (13).

One additional reason for our proposal was that figure 8 molecules were expected to sediment anomalously (in between bands) in propidium diiodide–CsCl gradients, i.e., their rotational constraints would not allow free and rapid conversion from a supercoiled to a fully relaxed figure 8 after nicking, under the conditions used (J. Vinograd, personal communication).

*An Electron Microscope Assay for Recombinant DNA Molecules.* A φX174 DNA structure that is recombinant is shown in Fig. 1a. This structure appears to contain two parental genomes which are linked in a figure 8 configuration. This structure was isolated from a mixed infection with *am3*(E) and $delE_{25}$, two genotypes that can be distinguished by their unequal contour lengths (1.70 μm and 1.55 μm, respectively). Figure 8 structures also can contain two *am3*(E) genomes (Fig. 1b) or two $delE_{25}$ genomes (Fig. 1c). The proposed structure of one type of figure 8 molecule is drawn in Fig. 1d, and examples of this structure are shown in Fig. 1e and f.

Figure 8 structures were observed to contain two parental genotypes in over 60% of the molecules measured, as shown in Fig. 2. Therefore, we conclude that figure 8 structures can and often do contain two parental genotypes. The apparent excess of recombinant figure 8's containing two genotypes is unexplained, but may arise from the fact that the two genomes were not completely homologous. Thus recombinant figure 8's containing two genotypes might have been preferentially "trapped" or stabilized.

*Frequency of Occurrence of Figure 8 Structures.* Since formation of φX174 recombinants by the major pathway requires

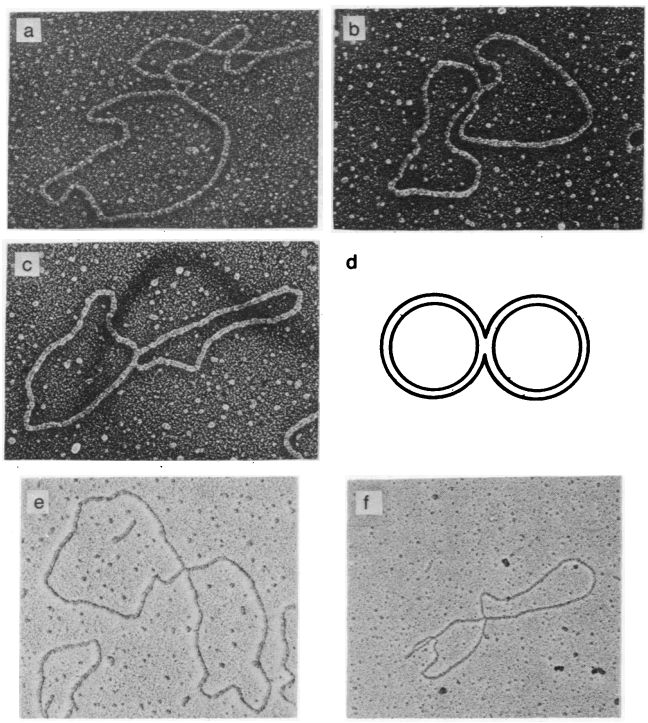


FIG. 1. Electron micrographs of φX174 DNA structures that are recombinant, spread by the aqueous Kleinschmidt procedure of Davis *et al.* (30). (a) A figure 8 structure containing one *am3*(E) and one $delE_{25}$ genome. The ratio of the two contour lengths is $1.08_3$. (b) A figure 8 structure containing two *am3*(E) genomes; the contour lengths are 1.69 μm. (c) A figure 8 structure containing two $delE_{25}$ genomes, formed in a cell-free system. The contour lengths are 1.56 μm. (d) Proposed structure of a figure 8 molecule. (e) A figure 8 structure formed *in vivo*. (f) A figure 8 structure formed artificially from a dimeric (−) strand and 2 wild-type (+) strands. Note that only (e) and (f) correspond to the structures observed by Gordon *et al.* (17). Our classification includes three types of figure 8 as illustrated above; approximately 40% are the type seen in (a), 20% the type seen in (b) and (c), and 40% the type seen in (e) and (f).

the host *recA*$^+$ allele (6, 7) (Table 1), we examined the frequency at which figure 8 structures could be detected in replicative form preparations grown in *recA*$^+$ and *recA*$^-$ cells. As shown in Table 2, the frequency of observed figure 8 structures was at least 10 fold greater in each of two different *recA*$^+$ strains, than in the *recA*$^-$ strain. This suggested that the formation of figure 8 molecules and of genetic recombinants were both controlled by the host *recA*$^+$ allele.

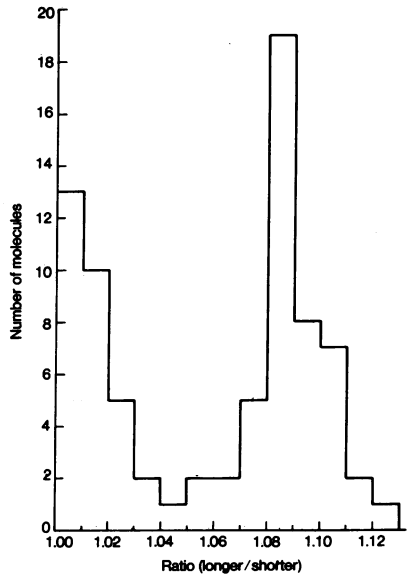


FIG. 2. The ratio of contour length of the longer of two monomer rings in a figure 8 to the contour length of the shorter monomer. Figure 8 molecules were defined as molecules which appeared to be 1:1 catenanes in the electron microscope, exhibited one-point attachments, and whose ratio of contour lengths did not exceed 1.15.

It is of considerable interest that figure 8 structures were observed to be formed in sonicates of *recA*$^+$ cells at a much higher frequency than in sonicates of *recA*$^-$ cells (Table 2). This suggests that figure 8 formation can be used as an *in vitro* assay for the *recA*$^+$ protein.

*How Are Recombinant Figure 8 Structures Formed?* We propose that the observed recombinant figure 8 structures were formed by the mechanism outlined in Fig. 3.

The postulated sequence of events is as follows: two single-stranded parental genomes (Fig. 3a) infect and enter the cell, forming double-stranded parental RF DNA molecules (Fig. 3b) (28), which are attached to the host cell membrane at an essential site (29). At some time early in infection (7), a single-strand break occurs or is introduced into one of the two parental genomes (11) (Fig. 3c). This break may be random or specific, natural or artificial, a few or many nucleotides long (11).

We now propose that "single-strand aggression" (11) results in the formation of figure 8 structures as shown in Fig. 3d and e. Formally, a figure 8 structure is an inescapable consequence of two circular genomes that undergo a re-

TABLE 2. *Frequency of occurrence of figure 8 molecules*

| Strain | Figure 8 molecules | | Circular multiple length | | Catenated multiple length | | Total number molecules examined |
|---|---|---|---|---|---|---|---|
| | Number | Frequency* × $10^3$ | Number | Frequency* × $10^3$ | Number | Frequency* × $10^3$ | |
| HF4714 *rec*$^+$ | 49 | 9.8 | 119 | 24 | 87 | 17 | ~5,000 |
| HF4712 *recA*$^-$ | 3 | 0.6 | 137 | 27 | 24 | 4.8 | ~5,000 |
| HF4704 *rec*$^+$ | 16 | 16 | 27 | 27 | 19 | 19 | ~1,000 |
| Starting material | 0 | <1 | 7 | 7 | 2 | 2 | ~1,000 |
| HF4714 *rec*$^+$ sonicate | 26 | 6.5 | 24 | 6 | 19 | 3.2 | ~4,000 |
| HF4712 *recA*$^-$ sonicate | 1(?) | $0.25 \times 10^{-4}$ | 36 | 9 | 7 | 1.8 | ~4,000 |

* Number of molecules found per total number of molecules examined.

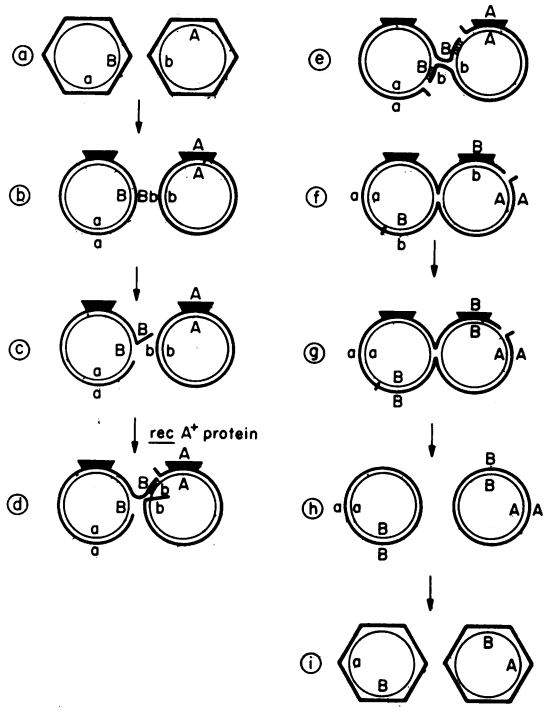


FIG. 3. Formation of figure 8 DNA molecules and $\phi$X174 recombinants. (a) Infection with two single-stranded parental genotypes (10). Note that A/a allele is drawn with a different orientation in each of the two molecules. This simplifies the later diagrams. (b) Parental RF formation (28) at essential bacterial site (29). (c) A single-strand break (10). (d) Single-strand aggression (11) catalyzed by *recA* protein (33). A figure 8 is formed with a unitary-crossed strand exchange (34). (e) A figure 8 with two mismatches is formed, as is a double-crossed strand exchange (34). (f) Double-strand branch migration (19, 20). (g) Heteroduplex correction (30, 35); this step occurs any time after d and prior to h. (h) Homozygous progeny RF formation; not at essential bacterial site (10). (i) Single-stranded recombinant and parental genotype (7, 36). DNA polymerase I, ligase, and putative endonuclease activities on the above molecules are omitted for clarity. For example, the gap and the extended single strand in 3e would presumably be closed, or excised and closed, respectively.

combination event involving a single strand. However, if we assume, as seems likely, that the number of base pairs participating in "single-strand aggression" is small (perhaps as few as 3), then such figure 8 structures would be rather unstable. Therefore we also invoke double-strand "branch migration" to stabilize the figure 8 structures (Fig. 3f).

No further assumptions are made. It has been shown previously by Weisbeck and van de Pol (30) and by Baas and Jansz (31, 32) that repair of mismatched base pairs occurs in $\phi$X174 heteroduplexes as shown in Fig. 3g. It has been shown previously by Doniger *et al.* (15) that recombinant multiple length molecules replicate and usually generate nonreciprocal recombinants as shown in Fig. 3h and i.

An interesting corollary of our proposal is that nonreciprocal recombinants must predominate by at least a 2:1 ratio if recombination proceeds through these figure 8 intermediates (Benbow and Sinsheimer, to be published).

## DISCUSSION

The purpose of this paper is 2-fold: to establish that figure 8 structures are intermediates in, or end products of, $\phi$X174 recombinant formation, and to propose a mechanism by which recombinant figure 8 structures can be formed.

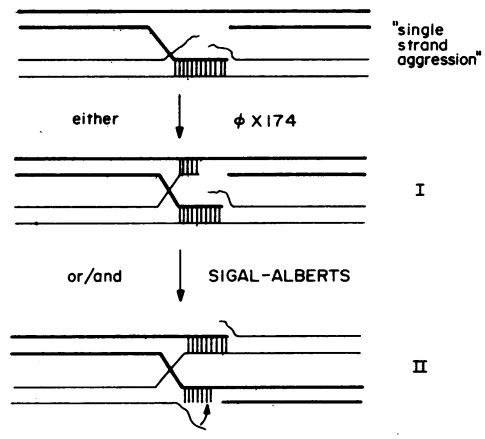


FIG. 4. Formation of a double-crossed strand exchange. A unitary-crossed strand aggression as shown in Fig. 3d. The region of the unitary exchange is redrawn in Fig. 4. The aggressive genome is indicated by solid thick lines; the invaded genome by solid thin lines. For $\phi$X174 (and many other genomes), the two genomes are closed circular. We have proposed in Fig. 3e that the displaced unpaired strand complementary to the region vacated by the aggressing strand will pair with that region (Alternative I). However, as first discussed by Sigal and Alberts (34), in any double-crossed strand exchange the outside strands and the bridging strands are interconvertible (by enantiomerization). We now suggest that unitary-crossed strand exchanges can undergo the same process, fortuitously leading to double-crossed strand exchanges. This process could occur in any unitary-crossed strand in which the base paired region is sufficiently stable (roughly 12 base pairs or more). It might be less likely to occur in $\phi$X174 because of the constraints introduced by the small circular genome (suggested by B. Alberts).

*(1) Figure 8 structures are recombinant DNA molecules of bacteriophage $\phi$X174.*

A role for figure 8 structures in $\phi$X174 recombinant formation was first proposed by Doniger *et al.* (15, 18) and by Benbow (10). Several direct and indirect lines of evidence support this conclusion.

*(i)* Figure 8 structures often contain two genotypes (Fig. 2). Since recombinant DNA molecules by definition contain genetic information from each of the parental genotypes, figure 8 structures must be considered "recombinant" in a formal sense. However, by itself this does not necessarily mean that they are relevant to $\phi$X174 recombinant formation.

*(ii)* Figure 8 molecules are formed in *recA*$^+$ cells or cell lysates at least 10 times more frequently than in *recA*$^-$ cells (Table 2). The host *recA*$^+$ allele is required for $\phi$X174 recombinant formation by the major pathway (6, 7) (Table 1), which indirectly suggests that the observed figure 8 structures are relevant to recombination.

*(iii)* Sedimentation procedures that enrich for recombinants also enrich for figure 8 structures (Table 1). We suggest that figure 8 structures are responsible for most of the 2- (Table 1) or 3- (15, 23) fold increases in recombinants found in multiple length DNA molecules.

*(iv)* Figure 8 structures are compatible with nonreciprocal recombination. We thus suggest that the recombinants observed in the single burst experiments of Doniger *et al.* (15) arise predominantly if not entirely from the 7% or more of figure 8 DNA molecules in their infecting material.

*(2) Figure 8 structures that are recombinant are generated by "single-strand aggression" and are stabilized by "branch migration."*

"Single-strand aggression" is a process catalyzed by the host *recA*$^+$ protein in which an RF DNA molecule containing a single strand region interacts with another RF DNA molecule, ultimately leading to a recombination event (11). The point we wish to emphasize here is that aggression by a *single* strand inevitably leads to a structure which, if stable, looks like a figure 8 in the electron microscope. In contrast, a circular double length recombinant molecule absolutely requires scissions in *both* strands. Conversely, *double*-strand breaks do not necessarily generate figure 8 molecules.

Since single- and double-strand "branch migration" occurs in φX174 molecules *in vitro* (10, 13, 21), we attribute our failure to detect figure 8 structures in *recA*$^-$ cells to a failure to form the nascent figure 8 molecules, rather than to a failure to "branch migrate" in these cells.

Our mechanism of figure 8 recombinant formation seems relevant not only to φX174 recombination but also to prokaryote recombination (φX recombination uses the host *recA*$^+$ protein), and to analogous processes in eukaryote recombination. However, most other organisms do not have small circular genomes; we, therefore, point out that our model in Fig. 3 applies to linear genomes (to draw, cut 180° from the b/B allele), resulting in chiasma (χ−) and H-branched structures (20). In addition, in linear or long circular genomes an alternative way to proceed from single-strand invasion to a double-strand exchange (i.e., from 3d to e) exists. This alternative, which is forbidden to φX174 because of its very short circular genome, is shown in Fig. 4.

The consequences of this alternative are striking. Reciprocal recombinants are generated for external markers with non-reciprocal (gene-convertible) regions of hybrid DNA in between.

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